The EMBO Journal Vol.16 No.7 pp.1647–1655, 1997

ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling

Diana Graus-Porta, Roger R.Beerli, John M.Daly and Nancy E.Hynes[1]

Friedrich Miescher Institute, CH-4002 Basel, Switzerland

[1]Corresponding author

We have analyzed ErbB receptor interplay induced by the epidermal growth factor (EGF)-related peptides in cell lines naturally expressing the four ErbB receptors. Down-regulation of cell surface ErbB-1 or ErbB-2 by intracellular expression of specific antibodies has allowed us to delineate the role of these receptors during signaling elicited by: EGF and heparin binding EGF (HB-EGF), ligands of ErbB-1; betacellulin (BTC), a ligand of ErbB-1 and ErbB-4; and neu differentiation factor (NDF), a ligand of ErbB-3 and ErbB-4. Ligand-induced ErbB receptor heterodimerization follows a strict hierarchy and ErbB-2 is the preferred heterodimerization partner of all ErbB proteins. NDF-activated ErbB-3 or ErbB-4 heterodimerize with ErbB-1 only when no ErbB-2 is available. If all ErbB receptors are present, NDF receptors preferentially dimerize with ErbB-2. Furthermore, EGF- and BTC-induced activation of ErbB-3 is impaired in the absence of ErbB-2, suggesting that ErbB-2 has a role in the lateral transmission of signals between other ErbB receptors. Finally, ErbB-1 activated by all EGF-related peptides (EGF, HB-EGF, BTC and NDF) couples to SHC, whereas only ErbB-1 activated by its own ligands associates with and phosphorylates Cbl. These results provide the first biochemical evidence that a given ErbB receptor has distinct signaling properties depending on its dimerization.

Keywords: ErbB receptors/lateral signaling/receptor dimerization/signal transduction/single chain antibody

Introduction

The ErbB family of receptor tyrosine kinases (RTKs) or subclass I RTKs comprises four members: epidermal growth factor receptor (EGFR)/ErbB-1 (Ullrich *et al.*, 1984), ErbB-2 (Yamamoto *et al.*, 1986), ErbB-3 (Kraus *et al.*, 1989; Plowman *et al.*, 1990) and ErbB-4 (Plowman *et al.*, 1993a). The four proteins are widely expressed in epithelial, mesenchymal and neuronal tissues and play fundamental roles during development (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). Interest in the ErbB family of RTKs is high due also to the involvement of some of its members in human cancers (Hynes and Stern, 1994; Salomon *et al.*, 1995).

Subclass I RTKs have an extracellular domain (ECD) which bears two cysteine-rich clusters and is responsible for interaction with polypeptide ligands. A direct consequence of ligand binding to the ECD is the formation of receptor dimers and stimulation of the intrinsic kinase activity, which leads to the phosphorylation of tyrosine residues in the intracellular domain of the receptors (van der Geer *et al.*, 1994). These serve as docking sites for a number of SH2- and PTB-domain containing proteins (Kavanaugh and Williams, 1994; Cohen,G.B. *et al.*, 1995) including the adaptor proteins SHC (Pelicci *et al.*, 1992) and Grb2 (Lowenstein *et al.*, 1992) and the p85 subunit of phosphatidylinositol (PtdIns) 3-kinase (Fedi *et al.*, 1994; Prigent and Gullick, 1994), which link RTKs to intracellular signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway (Egan and Weinberg, 1993) or the S6 kinase cascade (Ming *et al.*, 1994)

Regulation of ErbB receptor function is complex, since a large number of ligands, the EGF-related peptides, have been described. ErbB ligands can be classified into three groups and include: EGF and heparin binding EGF-like growth factor (HB-EGF), which bind ErbB-1 (Savage *et al.*, 1972; Higashiyama *et al.*, 1991); betacellulin (BTC), which is a ligand of ErbB-1 and ErbB-4 (Shing *et al.*, 1993; Beerli and Hynes, 1996; Riese *et al.* 1996); neu differentiation factors (NDFs)/heregulins (Peles and Yarden, 1993), which are ligands of ErbB-3 and ErbB-4 (Plowman *et al.*, 1993b; Carraway *et al.*; 1994), the respective low and high affinity receptors (Tzahar *et al.*, 1994).

By binding to the ECD of their respective receptors, EGF-related peptides induce not only receptor homodimers but also heterodimers. Consequently, although none of these peptides directly bind ErbB-2, all of them induce its tyrosine phosphorylation by triggering heterodimerization and cross-phosphorylation (King *et al.*, 1988; Plowman *et al.*, 1993b; Sliwkowski *et al.*, 1994; Beerli and Hynes, 1996). Cooperation of ErbB-2 with the other ErbB receptors has been reported (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996; Zhang *et al.*, 1996). Moreover, EGF and NDF receptors have been shown to compete for dimerization with ErbB-2 (Karunagaran *et al.*, 1995; Chen *et al.*, 1996). By means of intracellular expression of an endoplasmic reticulum (ER)-targeted single chain antibody (scFv) that leads to the specific and stable loss of cell surface ErbB-2 (Beerli *et al.*, 1994), we have previously shown that: (i) ErbB-2 enhances EGF-induced tyrosine phosphorylation of ErbB-1 and NDF-induced tyrosine phosphorylation of ErbB-3 and ErbB-4 (Beerli *et al.* 1995; Graus-Porta *et al.*, 1995); (ii) ErbB-2 potentiates and prolongs the signal transduction pathways elicited by EGF and NDF (Beerli *et al.*, 1995; Graus-Porta *et al.*; 1995; Karunagaran *et al.*, 1996). In addition, we and others have shown that ErbB-2 increases the affinity of both EGF and NDF for their

receptors (Wada *et al.*, 1990; Sliwkosky *et al.*, 1994; Karunagaran *et al.*, 1996). Together, these results suggest that ErbB-2 acts as a common receptor subunit of all the other ErbB proteins and that the physiological receptors for the EGF-related peptides are ErbB-2-containing heterodimers.

However, several recent observations apparently argue against this model. First, it has been suggested that ErbB-1–ErbB-3 heterodimers occur in cell lines overexpressing ErbB-1, where EGF can efficiently elevate tyrosine phosphorylation of ErbB-3 (Kim *et al.*, 1994; Soltoff *et al.*, 1994). Second, in cells that express moderate levels of the four proteins, EGF, HB-EGF and BTC not only activate their respective receptors and ErbB-2, but also ErbB-3 (Beerli and Hynes, 1996). Third, cooperative signaling of ErbB-3 and ErbB-4 with not only ErbB-2 but also ErbB-1 has been demonstrated when the receptors were expressed in cells devoid of ErbB proteins (Cohen,B.D. *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996; Zhang *et al.*, 1996). Finally, expression of different combinations of ErbB receptors in Ba/F3 hematopoietic cells has revealed that all heterodimers can be formed in response to the appropriate ligand (Riese *et al.*, 1995, 1996).

Expression of recombinant ErbB receptors in pairwise combinations is a viable approach to study their function. However, the four ErbB proteins are often co-expressed and this approach does not allow delineation of which ErbB receptor interactions are biologically relevant. Different heterodimers have been shown to elicit very different biological responses (Riese *et al.*, 1995, 1996), implying the recruitment and activation of distinct signaling molecules. Thus, it is crucial to elucidate which heterodimers are formed in a natural cellular context and to characterize their signaling properties.

To understand in more detail ligand-induced ErbB receptor heterodimerization and transactivation, as well as the consequent diversification of intracellular cascades, we have down-regulated the cell surface expression of ErbB-1 and ErbB-2 in a number of cell lines that co-express various amounts of the four ErbB proteins. To do so, ErbB-2-specific scFv-5R (Beerli *et al.*, 1994) has been expressed in T47D (Graus-Porta *et al.*, 1995) and A431 cells, leading to a complete loss of cell surface ErbB-2 and to its functional inactivation. ErbB-1-specific scFv-R1R has been expressed in T47D cells (Jannot *et al.*, 1996), leading to a dramatic reduction in cell surface ErbB-1. This approach has enabled us to analyze in more detail EGF-, HB-EGF-, BTC- and NDF-induced signaling and to unravel a hierarchy guiding the ligand-induced coordinated action of ErbB receptors.

Results

scFv-mediated intracellular retention of ErbB-1 results in impaired ErbB-2 activation by EGF agonists but not by NDF

To investigate in more detail ligand-induced ErbB receptor interactions, cell surface expression of either ErbB-1 or ErbB-2 was selectively down-regulated in T47D cells by intracellular expression of scFv-R1R (Jannot *et al.*, 1996) or scFv-5R (Beerli *et al.*, 1994, 1995; Graus-Porta *et al.*, 1995) respectively. Interestingly, intracellular retention of

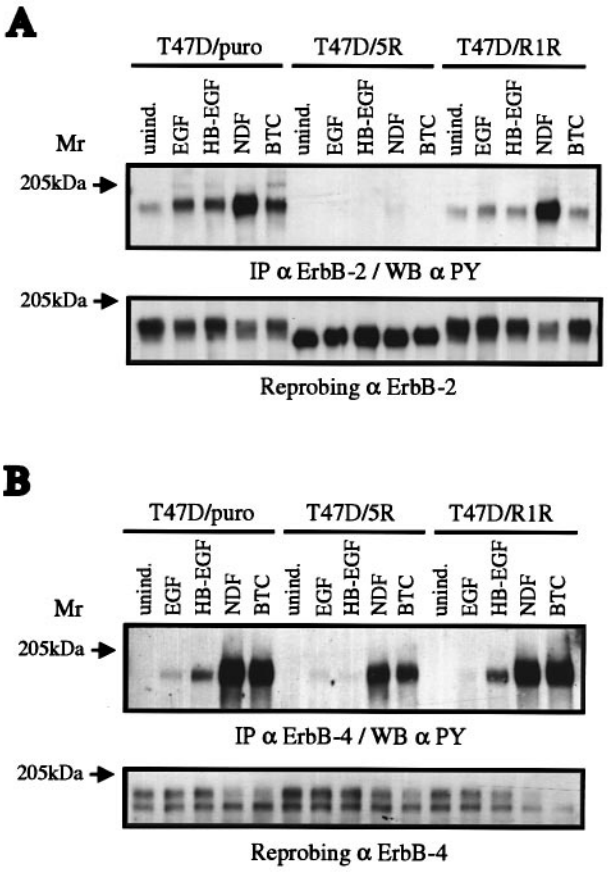

Fig. 1. Ligand-induced tyrosine phosphorylation of ErbB-2 and ErbB-4. T47D/puro, T47D/5R and T47D/R1R cells were starved for 24 h in serum-free medium. Prior to lysis, the cells were either treated with 4 nM indicated factors for 10 min or left untreated. ErbB-2 (**A**) and ErbB-4 (**B**) were immunoprecipitated from 200 μg and 2 mg total protein respectively, subjected to SDS–PAGE (7% gel) and analyzed by Western blotting with a phosphotyrosine-specific mAb (upper panels). Filters were stripped and reprobed with ErbB-2- and ErbB-4-specific antibodies (lower panels) respectively. The results shown are typical and representative of at least two independent experiments.

ErbB-1 but not ErbB-2 resulted in increased expression levels (Figures 1A and 4A), possibly due to an altered turnover of the ER-retained protein. We previously made a similar observation with the ER retention of a point-mutated, constitutively active ErbB-2 (Beerli *et al.*, 1994). We analyzed ErbB-2 activation by EGF-related peptides in T47D/R1R and T47D/5R sublines, as well as in T47D/puro vector control cells (Figure 1A). EGF, HB-EGF, BTC and NDF activated ErbB-2 in the control cells but not in T47D/5R cells, due to its disappearance from the plasma membrane. In T47D/R1R cells, displaying dramatically reduced levels of cell surface ErbB-1 (Jannot *et al.*, 1996), the EGF agonists did not significantly stimulate ErbB-2, whereas NDF led to phosphotyrosine levels comparable with those in the control cells. This is in accordance with the fact that NDF neither binds nor activates ErbB-1 in cells that co-express the four ErbB proteins (Figure 4A; Beerli and Hynes, 1996).

ErbB-2 enhances ErbB-4 tyrosine phosphorylation induced by EGF-related peptides

Various EGF-related peptides stimulate ErbB-4 tyrosine phosphorylation in T47D cells, the most efficient ones being

the ErbB-4 ligands BTC and NDF (Beerli and Hynes, 1996). To study the mechanism of its activation, we examined the involvement of ErbB-1 and ErbB-2 in stimulation of ErbB-4 phosphorylation (Figure 1B). Intracellular retention of ErbB-2 resulted in impaired activation of ErbB-4 by BTC and NDF, suggesting that activation of this receptor by its ligands involves ErbB-2-containing heterodimers. Moreover, EGF- and HB-EGF-induced tyrosine phosphorylation of ErbB-4 was also impaired in T47D/5R cells, demonstrating that even activation of ErbB-4 by ErbB-1 ligands involves ErbB-2. In accordance with the fact that BTC and NDF directly interact with ErbB-4, down-regulation of cell surface ErbB-1 did not affect ErbB-4 activation by these ligands, while it abolished activation of ErbB-4 by EGF. Surprisingly, HB-EGF was able to regulate ErbB-4 tyrosine phosphorylation in an ErbB-1-independent manner, suggesting that an additional receptor for this ligand must exist.

ErbB-2 enhances ErbB-3 tyrosine phosphorylation and association with p85

NDFs, the ErbB-3 ligands, activate ErbB-3 in diverse cell lines, and ErbB-2 dramatically enhances this effect (Beerli *et al.*, 1995; Graus-Porta *et al.*, 1995). Treatment of cells with EGF and BTC also results in increased ErbB-3-tyrosine phosphorylation (Beerli and Hynes, 1996). Since both peptides bind and activate ErbB-1 and stimulate ErbB-2 tyrosine phosphorylation, we examined whether scFv-mediated ER retention of either of the two receptors would interfere with ErbB-3 activation (Figure 2A). When compared with control cells, the EGF- and BTC-induced tyrosine phosphorylation of ErbB-3 was dramatically impaired in T47D/5R cells, identifying ErbB-2 as the mediator of ErbB-3 activation induced by both peptide factors. Whilst EGF did not activate ErbB-3 in T47D/R1R cells, NDF and BTC still did so, indicating that ErbB-1 is not required for ErbB-3 activation in response to these ligands.

ErbB-3 has been shown to efficiently associate with the p85 subunit of PtdIns 3-kinase, due to the presence of several consensus motifs in the receptor intracellular domain (Fedi *et al.*, 1994; Prigent and Gullick, 1994). Moreover, ER retention of ErbB-2 dramatically impairs NDF-induced recruitment of p85 (Beerli *et al.*, 1995). Therefore, we examined whether EGF-, HB-EGF- and BTC-induced ErbB-3 coupling to p85 was affected in cells lacking ErbB-2 (Figure 2B). Complex formation of p85 with ErbB-3 was diminished in response to all the peptides tested in T47D/5R cells. This result parallels the reduced tyrosine phosphorylation levels of ErbB-3 in these cells and corroborates the role of ErbB-2 in mediating ErbB-3 activation not only by NDF, but also by the other ErbB receptors ligands, EGF, HB-EGF and BTC.

It has been suggested that EGF-induced tyrosine phosphorylation of ErbB-3 occurs by direct ErbB-1/ErbB-3 crosstalk in A431 human carcinoma cells (Soltoff *et al.*, 1994). A431 cells have 1×10^6 EGFR molecules, whereas T47D cells have only 7000 molecules (Jannot *et al.*, 1996). It would be reasonable to assume that due to the high receptor density in A431 cells, EGF would trigger ErbB-1–ErbB-3 heterodimers and thereby activate ErbB-3 in an ErbB-2 independent manner. To examine this directly, A431 cells were infected with a retrovirus encoding scFv-5R, as well as with a vector control virus, giving rise to the sublines A431/5R and A431/puro (Figure 3A). Intracellular expression of ErbB-2-specific scFv in A431 cells (Figure 3A, left panel) resulted in the disappearance of ErbB-2 from the plasma membrane (our unpublished data) and in an increased electrophoretic mobility (Figure 3A, right panel). The A431 derivatives were treated with different ErbB ligands and ErbB-3 tyrosine phosphorylation was examined (Figure 3B). Activation of ErbB-3 observed in the control cells was reduced in A431/5R cells, demonstrating that ErbB-2 mediates, at least in part, ErbB-3 activation by these ligands even in cells expressing high levels of ErbB-1.

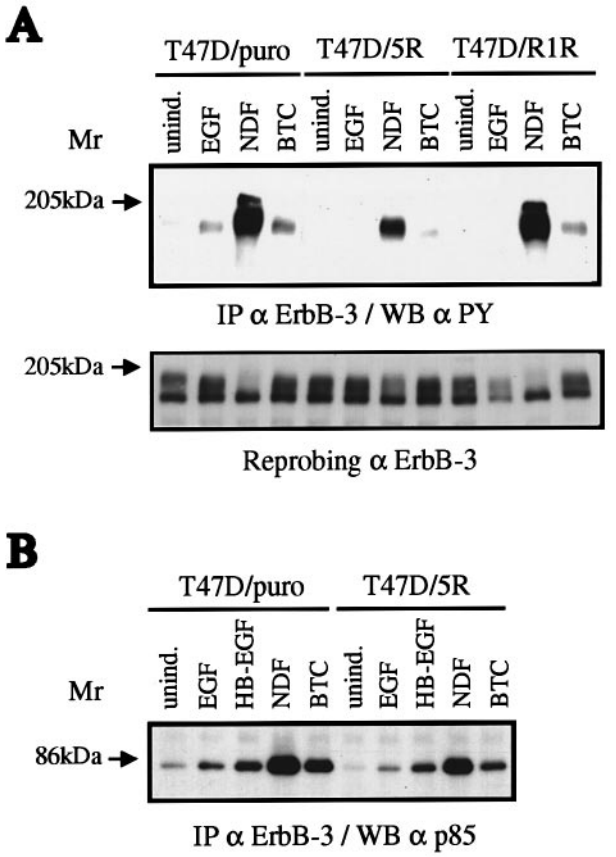

Fig. 2. Ligand-induced ErbB-3 tyrosine phosphorylation and complex formation with p85. The indicated cell lines were starved for 24 h in serum-free medium and, prior to lysis, were treated with 4 nM peptides for 10 min or left untreated. ErbB-3 was immunoprecipitated from 1 mg total protein and immunocomplexes were subjected to SDS–PAGE (7% gel) and analyzed by Western blotting with either (**A**, upper panel) a phosphotyrosine-specific mAb or (**B**) a p85-specific antibody. In (A) the filter was stripped and reprobed with ErbB-3-specific antibody (lower panel). The results shown are typical and representative of three independent experiments.

To confirm that activation of ErbB-3 by EGF occurs via a direct interaction between ErbB-2 and ErbB-3, we used the ErbB-2 overexpressing human mammary carcinoma cell line SKBR3. Cells treated with EGF showed increased levels of ErbB-2 co-immunoprecipitating with ErbB-3, indicating a direct interaction between ErbB-2 and ErbB-3 in response to EGF (Figure 3C).

ErbB-2 differentially modulates ErbB-1 activation by EGF-related peptides

All EGF agonists induce ErbB-1 tyrosine phosphorylation in T47D mammary epithelial cells (Beerli and Hynes, 1996)

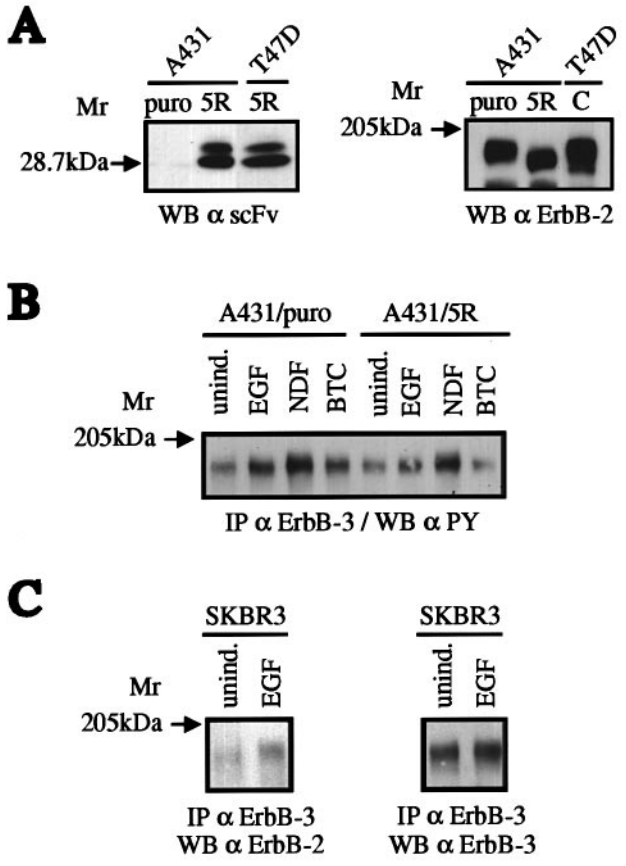

Fig. 3. (**A**) Expression of scFv-5R and ErbB-2 in A431/puro and A431/5R cells. (Left) Aliquots of 50 μg total protein were subjected to SDS–PAGE (15% gel), blotted and analyzed for expression of scFv-5R with an scFv-specific antiserum. (Right) Aliquots of 100 μg total protein were subjected to SDS–PAGE (7.5% gel), blotted and analyzed for ErbB-2 expression with 21N antiserum. C, control cells. (**B**) Ligand-induced ErbB-3 tyrosine phosphorylation. A431 derivative cell lines were serum starved for 24 h and, prior to lysis, treated with 4 nM of one of the indicated peptide ligands for 10 min or left untreated. ErbB-3 was immunoprecipitated from 1 mg total protein and immunocomplexes were subjected to SDS–PAGE (7% gel) and analyzed by Western blotting with a phosphotyrosine-specific mAb. The results shown are typical and representative of two independent experiments. (**C**) EGF-induced ErbB-2–ErbB-3 interaction. SKBR3 cells were serum starved for 24 h and, prior to lysis, treated with 10 nM EGF for 10 min or left untreated. ErbB-3 was immunoprecipitated from 1 mg total protein and immunocomplexes were subjected to SDS–PAGE (7% gel) and analyzed by Western blotting with ErbB-2-specific antiserum (left). The filter was stripped and reprobed with ErbB-3-specific antibody (right).

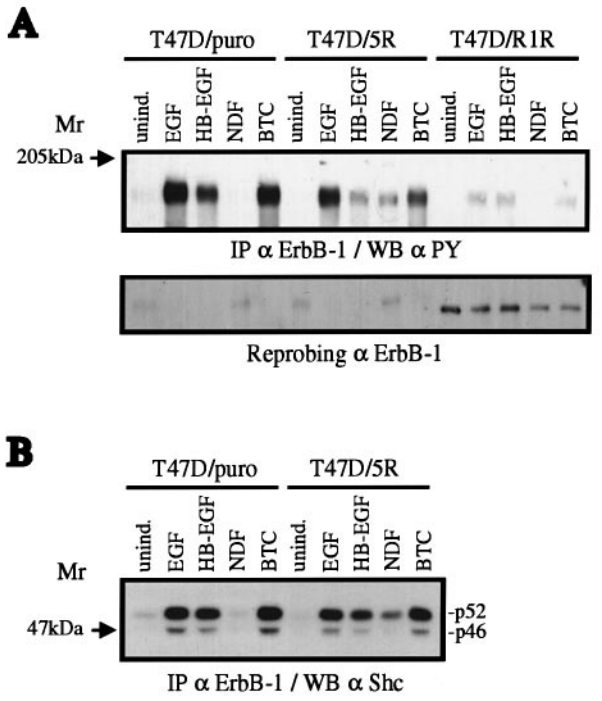

Fig. 4. Ligand-induced ErbB-1 tyrosine phosphorylation and complex formation with SHC. The indicated cell lines were serum starved for 24 h and, prior to lysis, treated with 4 nM diverse peptides for 10 min or left untreated. ErbB-1 was immunoprecipitated from 1 mg total protein and immunocomplexes were subjected to SDS–PAGE (7% gel) and analyzed by Western blotting with (**A**, upper panel) a phosphotyrosine-specific mAb or (**B**) an SHC-specific antibody. Bars indicate two of the three SHC isoforms. In (A) the filter was stripped and reprobed with ErbB-1-specific antibody 15E (lower panel). The results shown are typical and representative of three independent experiments.

and ErbB-2 enhances the EGF effect (Graus-Porta *et al.*, 1995). Thus, we examined whether activation of ErbB-1 by two other EGF agonists, HB-EGF and BTC, was also dependent upon the presence of ErbB-2 in the plasma membrane (Figure 4A). In T47D/5R cells, stimulation with both ligands led to lower levels of ErbB-1 tyrosine phosphorylation than in the control cells, indicating that ErbB-1–ErbB-2 heterodimers are also involved in ErbB-1 activation in response to HB-EGF and BTC.

Various reports have shown NDF-induced ErbB-1 activation when it was co-expressed with either ErbB-3 or ErbB-4 (Riese *et al.*, 1995; Cohen,B.D. *et al.*, 1996; Zhang *et al.*, 1996). However, we were unable to detect ErbB-1 tyrosine phosphorylation in response to NDF in cell lines naturally co-expressing the four ErbB receptors, including T47D, MCF7, MCF10A, OVCAR3 and A431 cells (Beerli and Hynes, 1996; our unpublished data). Thus, we investigated whether ErbB-2 interferes with this event by examining ErbB-1 activation in the presence and absence of ErbB-2 (Figure 4A). Indeed, NDF was able to activate ErbB-1 in the absence of ErbB-2, revealing that ErbB receptor interactions are hierarchical in nature. Thus, ErbB-2 is the preferred heterodimerization partner of NDF receptors and thereby prevents activation of ErbB-1 in a natural cellular context.

The SH2/PTB domain-containing adaptor protein SHC has been shown to couple RTKs to the Ras/MAPK pathway by interacting with specific phosphorylated tyrosines on the receptors (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992). We therefore examined whether ErbB-2 affects the ligand-induced ErbB-1 interaction with SHC (Figure 4B). All ErbB-1 ligands induced complex formation of ErbB-1 with SHC in control cells, which was reduced in T47D/5R cells, consistent with diminished tyrosine phosphorylation of the receptor. The opposite observation was made with NDF, since it could promote ErbB-1–SHC association only in the absence of ErbB-2. This is in accordance with NDF-induced tyrosine phosphorylation of ErbB-1 and demonstrates that NDF-activated ErbB-1 is a functional receptor able to couple to downstream signaling molecules.

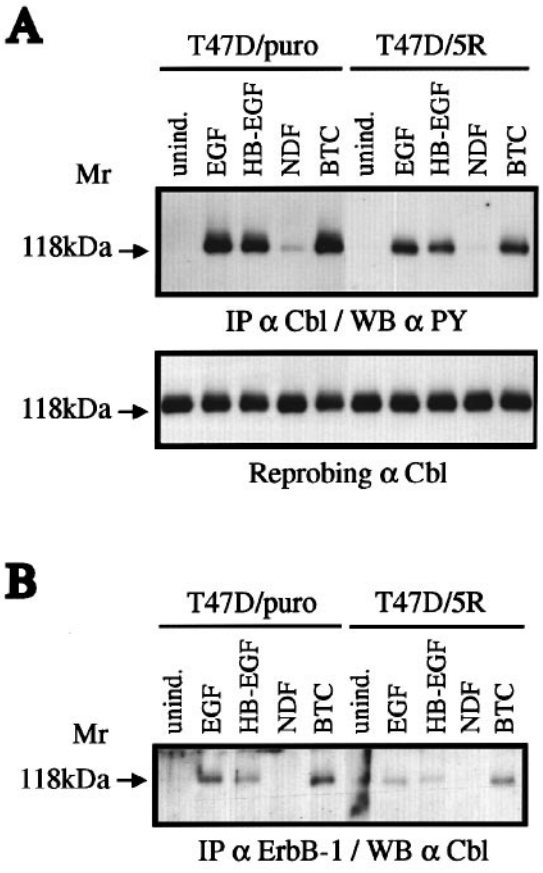

Fig. 5. Ligand-induced Cbl tyrosine phosphorylation and interaction with ErbB-1. T47D/puro and T47D/5R cells were serum starved for 24 h and, prior to lysis, treated for 10 min with 4 nM of one of the indicated ligands or left untreated. (**A**) Cbl was immunoprecipitated from 1 mg total protein, subjected to SDS–PAGE (8% gel) and analyzed by Western blotting with a phosphotyrosine-specific mAb (upper panel). The filter was stripped and reprobed with Cbl-specific antibody (lower panel). (**B**) ErbB-1 was immunoprecipitated from 1 mg total protein and the immunocomplexes were subjected to SDS–PAGE (8% gel) and analyzed by Western blotting with a Cbl-specific antibody. The results shown are typical and representative of two independent experiments.

scFv-mediated intracellular retention of ErbB-2 results in impaired ligand-induced coupling to Cbl

Stimulation of ErbB-1 (Galisteo *et al.*, 1995), but not of other ErbB receptors (Levkovitz *et al.*, 1996), has been shown to result in tyrosine phosphorylation and association with the downstream substrate Cbl. This prompted us to analyze Cbl tyrosine phosphorylation and complex formation with ErbB-1 in response to diverse ligands in the presence and absence of ErbB-2 (Figure 5). In T47D/puro control cells, the EGF agonists, but not NDF, significantly induced Cbl tyrosine phosphorylation, in agreement with their ability to stimulate ErbB-1. This was reduced in T47D/5R cells in accordance with the reduced activation of ErbB-1 (Figure 5A). Ligand-induced tyrosine phosphorylation of Cbl was paralleled by its co-immunoprecipitation with ErbB-1, which was also impaired in the absence of ErbB-2 (Figure 5B). Unexpectedly, after NDF treatment of T47D/5R cells Cbl was neither tyrosine phosphorylated (Figure 5A) nor co-immunoprecipitated with ErbB-1 (Figure 5B). It is noteworthy that a similar extent of ErbB-1 activation in response to HB-EGF was sufficient to promote phosphorylation of Cbl as well as its association with ErbB-1. These results suggest that NDF-activated ErbB-1 has signaling properties distinguishable from those of ErbB-1 activated by EGF agonists.

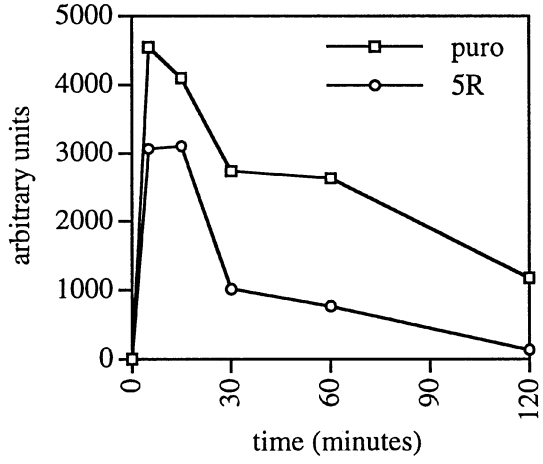

Fig. 6. Time course of BTC-induced ERK1 activation. The T47D sublines T47D/puro and T47D/5R were starved in serum-free medium for 24 h and, prior to lysis, treated with 1 nM BTC for the indicated times. The MAPK isoform ERK1 was immunoprecipitated from 200 μg cell lysate and *in vitro* kinase assays were performed using MBP as substrate. Proteins were subjected to SDS–PAGE (15% gel), blotted and phosphorylation of MBP was quantitated with a PhosphorImager.

scFv-mediated intracellular retention of ErbB-2 impairs BTC-dependent activation of MAPK

Activated RTKs stimulate MAPK by interacting with adaptor proteins like SHC and Grb2, which then couple to the Ras/MAPK pathway (Rozakis-Adcock *et al.*, 1992). We have previously shown that ErbB-2 modulates activation of MAPK by the ErbB-1 ligand EGF as well as by the ErbB-3 and ErbB-4 ligand NDF (Beerli *et al.*, 1995; Graus-Porta *et al.*, 1995). BTC is the prototype member of a new class of ligands, since it binds both ErbB-1 and ErbB-4. Thus, we investigated the involvement of ErbB-2 in BTC-triggered MAPK activation (Figure 6). BTC rapidly activated the MAPK isoform ERK1 in both T47D sublines. However, in scFv-5R-expressing cells the fold induction was reduced and activity returned to basal levels much more rapidly then in the control cells. Therefore, we conclude that the presence of ErbB-2 enhances and prolongs MAPK activity in response to the three different classes of EGF-related peptides, those binding ErbB-1, those binding ErbB3 and ErbB-4, and those binding ErbB-1 and ErbB-4.

Discussion

In this study we have analyzed EGF-, HB-EGF-, BTC- and NDF-induced signaling in several human epithelial cell lines co-expressing all four presently known ErbB receptors. Single chain antibody-mediated down-regulation of cell surface ErbB-1 and ErbB-2 has allowed us to discover novel aspects of ligand-induced ErbB receptor interplay. First, ErbB receptor interactions induced by various EGF-related growth factors are less diverse than previously suggested (Cohen,B.D. *et al.*, 1996; Riese *et al.*, 1995, 1996; Zhang *et al.*, 1996) and follow a strict

hierarchy, with ErbB-2 being the preferred heterodimerization partner for all other ErbB proteins. Second, ErbB-2 is involved in horizontal signaling, mediating the lateral transmission of signals between ErbB receptors. Third, ErbB-2 enhances and prolongs the MAPK signaling cascade in response not only to EGF and NDF (Graus-Porta *et al.*, 1995) but also in response to BTC. Fourth, the data presented in this paper demonstrate that a given ErbB receptor can acquire different signaling properties depending on its dimerization partner. Finally, our results suggest that ErbB-1 is not the only receptor for HB-EGF.

NDF binds and activates ErbB-3 and ErbB-4, triggers ErbB-2-containing heterodimers and induces ErbB-2 tyrosine phosphorylation (Carraway *et al.*, 1994). Moreover, the intracellular retention of ErbB-2 results in impaired NDF-induced activation of both receptors (Beerli *et al.*, 1995; Graus-Porta *et al.*, 1995). In contrast, we never observed an increase in ErbB-1 tyrosine phosphorylation in response to NDF, suggesting that no ErbB-1-containing heterodimers are formed. Moreover, in T47D cells depleted of ErbB-1, NDF activated ErbB-2, ErbB-3 and ErbB-4 to the same extent as in the control cells, indicating that ErbB-1 does not participate in NDF signaling. Other reports have presented evidence for NDF-induced formation of ErbB-3–ErbB-1 and ErbB-4–ErbB-1 heterodimers when these pairs of receptors were co-expressed in host cell lines that do not bear endogenous ErbB proteins (Riese *et al.*, 1995; Cohen,B.D. *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996; Zhang *et al.*, 1996). In accordance with these findings, NDF readily induced ErbB-1 tyrosine phosphorylation and association with the adaptor protein SHC in T47D cells devoid of cell surface ErbB-2. These results directly show, for the first time, that ligand-induced ErbB receptor heterodimerization follows a strict hierarchy. If no ErbB-2 is available, ErbB-3 and/or ErbB-4 are able to heterodimerize with ErbB-1 in response to NDF. However, if the four ErbB proteins are present on the plasma membrane, the NDF receptors preferentially dimerize with ErbB-2.

EGF has been found to activate ErbB-3 in cell lines overexpressing ErbB-1 (Kim *et al.*, 1994; Soltoff *et al.*, 1994). Moreover, co-expression of ErbB-1 with either ErbB-3 or ErbB-4 in cell types lacking endogeneous ErbB proteins allows EGF to regulate tyrosine phosphorylation of these receptors (Riese *et al.*, 1995; Cohen,B.D. *et al.*, 1996; Zhang *et al.*, 1996). These results imply that EGF may induce ErbB-1–ErbB-3 and ErbB-1–ErbB-4 heterodimers. However, our experiments demonstrate that the absence of functional ErbB-2 dramatically impairs EGF-triggered ErbB-3 and ErbB-4 tyrosine phosphorylation, as well as association of ErbB-3 with the p85 subunit of PtdIns 3-kinase. Significantly, efficient EGF-induced activation of ErbB-3 is dependent on ErbB-2 not only in T47D cells, which have low levels of ErbB-1 (7000 molecules/cell), but also in ErbB-1 overexpressing A431 cells (1×10^6 molecules/cell). Thus, formation of ErbB-1–ErbB-3 and ErbB-1–ErbB-4 heterodimers cannot account for activation of NDF receptors by EGF. Instead, it appears that EGF activates ErbB-3 and ErbB-4 via ErbB-2. Indeed, in SKBR3 cells an EGF-induced direct ErbB-2–ErbB-3 interaction was detected. It is known that dimerization of receptors is not a static, but rather a dynamic and reversible process (Yarden and Schlessinger, 1987). Thus, ErbB-2 may first dimerize with ErbB-1 in response to EGF and then, in its phosphorylated and activated state, be released and dimerize with and phosphorylate ErbB-3 or ErbB-4. Alternatively, the formation of ErbB receptor oligomers may occur (Lax *et al.*, 1991). Therefore, EGF-induced activation of ErbB-3 and ErbB-4 could be the result of receptor transphosphorylation occuring only within ErbB-1–ErbB-2–ErbB-3 or ErbB-1–ErbB-2–ErbB-4 trimers. In both models, ErbB-2 is required to activate NDF receptors in response to EGF. However, future studies will be required to delineate the precise mechanism of lateral transmission of signals.

BTC is a ligand for ErbB-1 and ErbB-4 (Beerli and Hynes, 1996; Riese *et al.*, 1996) and BTC-induced ErbB-1–ErbB-2 and ErbB-4–ErbB-2 heterodimers have been reported (Riese *et al.*, 1996). Consistent with this, down-regulation of ErbB-1 results in diminished ErbB-2 phosphorylation and the absence of cell surface ErbB-2 results in impaired BTC-induced ErbB-1 and ErbB-4 activation, suggesting that BTC also signals through ErbB-2-containing heterodimers. Although BTC could presumably induce ErbB-4–ErbB-1 heterodimers, down-regulation of ErbB-1 in T47D cells does not affect ErbB-4 activation, suggesting that ErbB-4–ErbB-2 heterodimers, and possibly ErbB-4 homodimers, are sufficient for BTC to activate ErbB-4. BTC was also able to elevate tyrosine phosphorylation of ErbB-3 when co-expressed with ErbB-1 in Ba/F3 cells, but not when co-expressed with ErbB-4, implying that at least ErbB-1–ErbB-3 heterodimers are possible (Riese *et al.*, 1996). However, in the human epithelial cell lines T47D and A431, BTC-induced tyrosine phosphorylation of ErbB-3 was dramatically impaired in the absence of ErbB-2, indicating that, by analogy with the ErbB-1 ligand EGF, BTC activation of ErbB-3 involves the interplay of three receptors, ErbB-1–ErbB-2–ErbB-3 and ErbB-4–ErbB-2–ErbB-3.

We show that down-regulation of cell surface ErbB-2 has dramatic consequences on downstream signaling events elicited by BTC. In particular, intracellular retention of ErbB-2 results in transient ligand-induced MAPK activity. We previously made a similar observation with EGF and NDF (Graus-Porta *et al.*, 1995), which correlated with a decreased ligand affinity due to an accelerated off-rate (Karunagaran *et al.*, 1996). Thus, it is reasonable to suspect that ErbB-2-containing heterodimers are not only the high affinity receptors for EGF and NDF, but for all EGF-related growth factors.

HB-EGF, although to a lesser extent than NDF and BTC, is also able to elevate tyrosine phosphorylation of ErbB-4 (Beerli and Hynes, 1996; Figure 1B). Binding of an EGF agonist to ErbB-1 could theoretically trigger ErbB-1–ErbB-4 dimers and subsequently activate ErbB-4. Nevertheless, in T47D cells depleted of ErbB-1, HB-EGF, but not EGF, still activated ErbB-4 to the same extent as in the control cells, which implies the existence of an additional receptor for HB-EGF. This result is reminiscent of BTC, making it possible that HB-EGF also binds ErbB-1 and ErbB-4. Alternatively, HB-EGF could bind ErbB-3, thereby triggering ErbB-3–ErbB-4 dimers. Indeed, HB-EGF activates ErbB-3 not only in T47D/puro but also in T47D/R1R cells (our unpublished data). In both cases, a requirement for ErbB-2 for HB-EGF activation of ErbB-4 becomes evident in T47D/5R cells. Further studies

will be required to elucidate the mechanism by which HB-EGF activates ErbB-4.

It is likely that the diversity of the biological responses triggered by the EGF-related ligands is due to the ability of each different ErbB family member to couple with distinct and specific intracellular signaling pathways (Di Fiore *et al.*, 1990; Taverna *et al.*, 1991; Riese *et al.*, 1995., Beerli and Hynes, 1996; Pinkas-Kramarski *et al.*, 1996). For instance, ErbB-3 differs from the other receptors in its ability to directly interact with p85 but not with phospholipase Cγ or GTPase activating protein (Fedi *et al.*, 1994). On the other hand, ErbB-1 seems to be the only ErbB receptor able to interact with and phosphorylate the Cbl proto-oncogene product (Levkowitz *et al.*, 1996). Moreover, the signal elicited by a receptor heterodimer is not simply defined by addition of the signaling properties of the individual dimerization partners (Alimandi *et al.*, 1995). Indeed, our results demonstrate that the ability of a specific ErbB protein to recruit downstream signaling molecules is dependent upon its dimerization partner. In particular, all EGF-related peptides induce ErbB-1 to interact with the adaptor protein SHC. However, only ErbB-1 ligands, which activate the receptor mainly by inducing ErbB-1 homodimers and ErbB-1–ErbB-2 heterodimers, but not NDF, which can only activate ErbB-1 by promoting heterodimers with ErbB-3 and ErbB-4, allow for detectable Cbl tyrosine phosphorylation and complex formation with ErbB-1. This result raises the possibility that ErbB-1 activated by NDF bears a pattern of phosphorylated sites or has a conformation distinct from ErbB-1 activated by EGF agonists.

In summary, the results presented in this paper allow us to propose a model of ErbB receptor function (Figure 7). According to this model, ligand-induced ErbB receptor dimerization is governed by a strict hierarchy, with ErbB-2 being the preferred heterodimerization partner of all other ErbB proteins. Thus, NDF receptors readily dimerize with ErbB-2 but not with ErbB-1 if all four receptors are present (Figure 7A, left). Interactions between NDF receptors and ErbB-1 are only favored in cells engineered to lack cell surface ErbB-2 (Figure 7A, right). Similarly, the receptors for EGF and BTC also preferentially interact with ErbB-2. However, in contrast to NDF receptors, ligand-activated EGF and BTC receptors have the capability to transactivate ErbB proteins distinct from ErbB-2. This could at least in part be due to direct crosstalk, as evidenced in cells lacking cell surface ErbB-2 (Figure 7B and C, right). However, the presence of ErbB-2 dramatically enhances transactivation of NDF receptors by ErbB-1, demonstrating that ErbB-2 is involved in lateral transmission of signals (Figure 7B and C, left). It is noteworthy that this signaling occurs in a directed manner, since NDF receptors did not transactivate ErbB-1 in the presence of ErbB-2 (Figure 4A). This suggests that NDF receptors form more stable complexes with ErbB-2 than does ErbB-1, as already previously suggested (Chen *et al.*, 1996).

In conclusion, the proposed model of ErbB receptor function provides a possible explanation for the high oncogenic potential of ErbB-2. Overexpression of ErbB-2 leading to constitutive activation of its kinase is observed in many human tumors and frequently correlates with more malignant disease (Hynes and Stern, 1994). Moreover, ErbB-2 is important not only for growth of ErbB-2

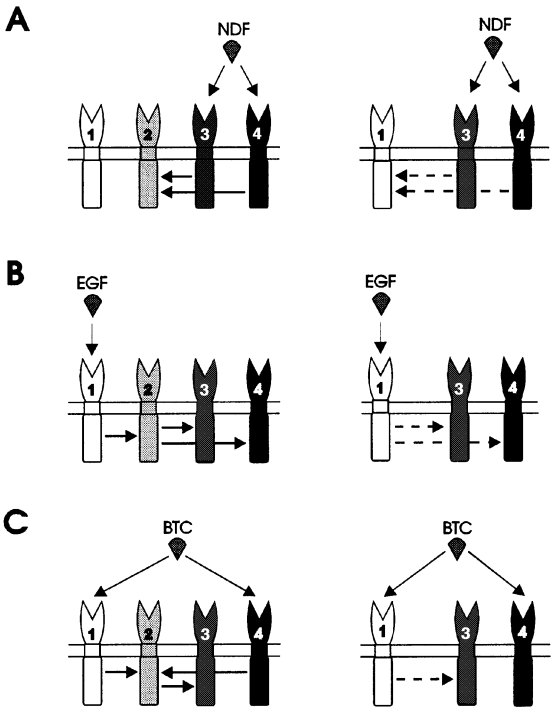

Fig. 7. Model of ErbB receptor interactions induced by EGF-related factors in the presence (left) or absence (right) of ErbB-2. ErbB receptor dimerization is a hierarchical process and ErbB-2 is the preferred heterodimerization partner of the receptors for NDF (**A**), EGF (**B**) and BTC (**C**). Activation of ErbB-2 in response to EGF and BTC, but not to NDF, allows lateral transmission of signals. Note that BTC has previously been shown not to induce ErbB-4–ErbB-3 dimers even in the absence of ErbB-2 (Riese *et al.*, 1996).

but also of ErbB-1 overexpressing tumor cells (Jannot *et al.*, 1996). Our results suggest that the remarkable transforming potency of ErbB-2 is due not only to its ability to heterodimerize with the other ErbB receptors, but also to its involvement in lateral signaling.

Materials and methods

Materials

Recombinant human EGF was from Sigma, recombinant human HB-EGF and BTC from R&D Systems. The recombinant human EGF-β1-like domain of NDF used in all the experiments was a gift from Amgen (Thousand Oaks, CA). Antibodies used were: EGFR-specific mAbs EGFR1 (Amersham) and 528 (Santa Cruz Biotechnology) and EGFR-specific antiserum 15E (Gullick *et al.*, 1985), ErbB-2-specific antiserum 21N (Hynes *et al.*, 1989), ErbB-3-specific affinity purified rabbit polyclonal antibody C17 (Santa Cruz Biotechnology), ErbB-4-specific mAb 111 (Chen *et al.*, 1996), ErbB-4-specific affinity purified rabbit polyclonal antibody C-18 (Santa Cruz Biotechnology), Cbl-specific affinity purified rabbit polyclonal antibody C15 (Santa Cruz Biotechnology), Shc-specific rabbit IgG (UBI), p85-specific antiserum (UBI), ERK1-specific antiserum (Marte *et al.*, 1995), phosphotyrosine-specific mAb (Druker *et al.*, 1989) and scFv-specific antiserum (Beerli *et al.*, 1994).

Intracellular expression of ErbB-2-specific scFv

Amphotropic virus encoding scFv-5R, as well as empty vector control virus, were used to infect the human vulval carcinoma cell line A431. Four days after infection the cells were subjected to selection in 2 μg/ml puromycin for 14 days. Pools of puromycin-resistant cells were analyzed in all the experiments.

Cell culture

T47D/puro, T47D/5R and T47D/R1R (Graus-Porta *et al.*, 1995; Jannot *et al.*, 1996) and A431/puro and A431/5R cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supple-

mented with 10% fetal calf serum (Gibco BRL), 5 μg/ml insulin (Sigma) and 1 μg/ml puromycin. SKBR3 cells were maintained in DMEM supplemented with 10% fetal calf serum. Prior to growth factor stimulation cells were starved for 24 h in serum-free medium [DMEM containing 1 mg/ml fetuin (Sigma) and 10 μg/ml transferrin (Sigma)].

Immunoprecipitation and Western blot analysis

Cells were solubilized in Triton extraction buffer (50 mM Tris, pH 7.5, 5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium molybdate, 20 μM phenylarsine oxide, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 10 min on ice. The lysates were clarified by centrifugation at 16 000 *g* for 15 min. For immunoprecipitations, equal amounts of protein were incubated with specific antibodies for 2 h on ice. Immune complexes were collected with protein A–Sepharose (Sigma), washed three times with lysis buffer and once with TNE buffer (50 mM Tris–HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA). Bound proteins were released by boiling in sample buffer. Total cell lysates or immunoprecipitates were subjected to SDS–PAGE and proteins were blotted onto polyvinylidene difluoride membranes. After blocking with 20% horse serum (Gibco BRL) in TTBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20), filters were probed with specific antibodies and proteins visualized with peroxidase-coupled secondary antibody using the ECL detection system (Amersham). In some experiments, filters were stripped in SDS buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.8) for 30 min at 65°C, washed three times in TTBS, blocked and reprobed with the indicated antibodies.

MAPK assay

Cells were stimulated at 37°C with 1 nM BTC for the indicated times and lysed in ELB (50 mM β-sodium glycerophosphate, 1.5 mM EGTA, 2 mM sodium orthovanadate, 1 mM dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM benzamidine and 1% v/v NP-40). The MAPK isoform ERK1 was immunoprecipitated from 200 μg total lysate with 2 μl specific antiserum (Marte *et al.*, 1995) and protein A–Sepharose. The immunoprecipitates were washed three times with ELB and once with kinase buffer (30 mM Tris–HCl, pH 8.0, 20 mM $MnCl_2$ and 2 mM $MgCl_2$). The activity of the MAPK isoform ERK1 was then determined by incubating the immune complexes at 37°C for 30 min with 30 μl kinase buffer containing 15 μg myeloid basic protein (MBP), 10 μM cold ATP and 0.1 μM [γ-^{32}P]ATP (1200 Ci/mmol). The reaction was stopped with sample buffer, proteins subjected to 15% SDS–PAGE, blotted and the phosphorylation of MBP was quantitated with a PhosphorImager (Molecular Dynamics).

Acknowledgements

We wish to thank Dr Doriano Fabbro for the ERK1-specific antiserum and Dr Dirk Moritz for the scFv-specific antiserum. We also thank Drs Brigitte Gay and Nicholas Pullen for their critical reading of the manuscript, as well as acknowledging the valuable technical suggestions of Dr Nicholas Pullen. We are grateful to Amgen (Thousand Oaks, CA) for providing us with recombinant human NDF. D.G.-P. was the recipient of a fellowship from the Krebsliga beider Basel. J.M.D. was the recipient of a fellowship from the Schweizerische Krebsliga.

References

Alimandi,M., Romano,A., Curia,M.C., Muraro,R., Fedi,P., Aaronson, S.A., Di Fiore,P.P. and Kraus,M.H. (1995) Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene*, **10**, 1813–1821.

Beerli,R.R. and Hynes,N.E. (1996) Epidermal growth factor-related peptides activate distinct subsets of ErbB receptors and differ in their biological activities. *J. Biol. Chem.*, **271**, 6071–6076.

Beerli,R.R., Wels,W. and Hynes,N.E. (1994) Intracellular expression of single-chain antibodies reverts ErbB-2 transformation. *J. Biol. Chem.*, **269**, 23931–23936.

Beerli,R.R., Graus-Porta,D., Woods-Cook,K., Chen,X., Yarden,Y. and Hynes,N.E. (1995) Neu differentiation activation of ErbB-3 and ErbB-4 is cell specific and displays a differential requirement for ErbB-2. *Mol. Cell. Biol.*, **15**, 6496–6505.

Carraway,K.L. *et al.* (1994) The *erbB*3 gene product is a receptor for heregulin. *J. Biol. Chem.*, **269**, 14303–14306.

Chen,X. *et al.* (1996) An immunological approach reveals biological differences between the two NDF/heregulin receptors, ErbB-3 and ErbB-4. *J. Biol. Chem.*, **271**, 7620–7629.

Cohen,B.D., Green,J.M., Foy,L. and Fell,H.P. (1996) HER4-mediated biological and biochemical properties in NIH 3T3 cells. *J. Biol. Chem.*, **271**, 4813–4818.

Cohen,G.B., Ren,R. and Baltimore,D. (1995) Modular binding domains in signal transduction proteins. *Cell*, **80**, 237–248.

Di Fiore,P.P., Segatto,O., Taylor,W.G., Aaronson,S.A. and Pierce,J.H. (1990) EGF receptor and *erb*B-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. *Science*, **248**, 79–83.

Druker,B.J., Mamon,H.J. and Roberts,T.M. (1989) Oncogenes, growth factors, and signal transduction. *New Engl. J. Med.*, **321**, 1383–1391

Egan,S.E. and Weinberg,R.A. (1993) The pathway to signal achievement. *Nature*, **365**, 781–783.

Fedi,P., Pierce,J.H., Di Fiore,P.P. and Kraus,M.H. (1994) Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase Cγ or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. *Mol. Cell. Biol.*, **14**, 492–500.

Galisteo,M.L., Dikik,I., Batzer,A.G., Langdon,W.Y. and Schlessinger,J. (1995) Tyrosine phosphorylation of the c-*cbl* proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation. *J. Biol. Chem.*, **270**, 20242–20245.

Gassmann,M., Casagranda,F., Orioli,D., Simon,H., Lai,C., Klein,R. and Lemke,G. (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature*, **378**, 390–394.

Graus-Porta,D., Beerli,R.R. and Hynes,N.E. (1995) Single-chain antibody-mediated intracellular retention of ErbB-2 impairs neu differentiation factor and epidermal growth factor signaling. *Mol. Cell. Biol.*, **15**, 1182–1191.

Gullick,W.J., Downward,D.J., Foulkes,J.G. and Waterfield,M.D. (1985) Antibodies to the ATP-binding site of the human epidermal growth factor (EGF) receptor as specific inhibitors of EGF-stimulated protein-tyrosine kinase activity. *EMBO J.*, **4**, 2869–2877.

Higashiyama,S., Abraham,J.A., Miller,J., Fiddes,J.C. and Klagsbrun,M. (1991) A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science*, **251**, 936–939.

Hynes,N.E. and Stern,D.F. (1994) The biology of *erbB-2*/*neu*/HER2 and its role in cancer. *Biochim. Biophys. Acta*, **1198**, 165–184

Hynes,N.E., Gerber,H.A., Saurer,S. and Groner,B. (1989) Overexpression of the c-erbB-2 protein in human breast tumor cell lines. *J. Cell. Biochem.*, **39**, 167–173

Jannot,Ch., Beerli,R.R., Mason,S., Gullick.W.J. and Hynes,N.E. (1996) Intracellular expression of a single-chain antibody directed to the epidermal growth factor receptor leads to growth inhibition of tumor cells. *Oncogene*, **13**, 275–282.

Karunagaran,D., Tzahar,E., Liu,N., Wen,D. and Yarden,Y. (1995) Neu differentiation factor inhibits EGF binding. *J. Biol. Chem.*, **270**, 9982–9990.

Karunagaran,D., Tzahar,E., Beerli,R.R., Chen,X., Graus-Porta,D., Ratzkin,B.J., Seger,R., Hynes,N.E. and Yarden,Y. (1996) ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.*, **15**, 254–264.

Kavanaugh,W.M. and Williams,L.T. (1994) An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science*, **266**, 1862–1865.

Kim,H.-H., Sierke,S.L. and Koland,J.G. (1994) Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the *erb*B3 gene product. *J. Biol. Chem.*, **269**, 24747–24755.

King,C.R., Borrello,I., Bellot,F., Comoglio,P. and Schlessinger,J. (1988) EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the *erb*B-2 protein in the mammary tumor cell line SK-BR-3. *EMBO J.*, **7**, 1647–1651.

Kraus,M.H., Issing,W., Miki,T., Popescu,N.C. and Aaronson,S.A. (1989) Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proc. Natl Acad. Sci. USA*, **86**, 9193–9197.

Lax,I., Mitra,A.K., Ravera,C., Hurwitz,D.R., Rubinstein,M., Ullrich,A., Stroud,R.M. and Schlessinger,J. (1991) Epidermal growth factor (EGF) induces oligomerization of soluble, extracellular, ligand-binding domain of EGF receptor. A low resolution projection stucture of the lignad-binding domain. *J. Biol. Chem.*, **266**, 13828–13833.

Lee,K.-F., Simon,H., Chen,H., Bates,B., Hung,M.-C. and Hauser,C. (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature*, **378**, 394–398.

Levkovitz,G., Klapper,L.N., Tzhar,E., Freywald,A., Sela,M. and Yarden,Y. (1996) Coupling of the c-Cbl protooncogene product to ErbB-1/EGF-receptor but not to other ErbB proteins. *Oncogene*, **12**,1117–1125

Lowenstein,E.J. *et al.* (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell*, **70**, 431–442.

Marte,B.M., Graus-Porta,D., Jeschke,M., Fabbro,D., Hynes,N.E. and Taverna,D. (1995) NDF activates MAP kinase and p70/p85 S6 kinase during proliferation or differentiation of mammary epithelial cells. *Oncogene*, **10**, 167–175.

Ming,X.-F., Burgering,B.M.T., Wennström,S., Claesson-Welsh,L., Heldin,C.-H., Bos,J.L., Kozma,S.C. and Thomas,G. (1994) Activation of p70/p85 S6 kinase by a pathway independent of $p21^{ras}$. *Nature*, **371**, 426–429.

Peles,E. and Yarden,Y. (1993) Neu and its ligands: from an oncogene to neural factors. *BioEssays*, **15**, 815–824.

Pelicci,G. *et al.* (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell*, **70**, 93–104.

Pinkas-Kramarski,R. *et al.* (1996) Diversification of neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.*, **15**, 2452–2467.

Plowman,G.D., Whitney,G.S., Neubauer,M.G., Green,J.M., McDonald, V.L., Todaro,G.J. and Shoyab,M. (1990) Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc. Natl Acad. Sci. USA*, **87**, 4905–4909.

Plowman,G.D., Culouscou,J.-M., Whitney,G.S., Green,J.M., Carlton, G.W., Foy,L., Neubauer,M.G. and Shoyab,M. (1993a) Ligand-specific activation of $HER4/p180^{erbB4}$, a fourth member of the epidermal growth factor receptor family. *Proc. Natl Acad. Sci. USA*, **90**, 1746–1750.

Plowman,G.D., Green,J.M., Culouscou,J.-M., Carlton,G.W., Rothwell, V.M. and Buckley,S. (1993b) Heregulin induces tyrosine phosphorylation of $HER4/p180^{erbB4}$. *Nature*, **366**, 473–475.

Prigent,S.A. and Gullick,W. (1994) Identification of c-erbB-3 binding sites for phosphatidylinositol 3′-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *EMBO J.*, **13**, 2831–2841.

Riese,D.J., van Raaij,T.M., Plowman,G.D., Andrews,G.C. and Stern,D.F. (1995) The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol. Cell. Biol.*, **15**, 5770–5776.

Riese,D.J., Bermingham,Y., van Raaij,T.M., Buckley,S., Plowman,G.D. and Stern,D.F. (1996) Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-β. *Oncogene*, **12**, 345–353.

Rozakis-Adcock,M. *et al.* (1992) Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in the activation of the Ras pathway by tyrosine kinases. *Nature*, **360**, 689–692.

Salomon,D.S., Brandt,R., Ciardiello,F. and Normanno,N. (1995) Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol. Hematol.*, **19**, 183–232.

Savage,C.R.,Jr, Inagami,T. and Cohen,S. (1972) The primary structure of epidermal growth factor. *J. Biol. Chem.*, **241**, 7612–7621.

Shing,Y., Christofori,G., Hanahan,D., Ono,Y., Sasada,R., Igarashi,K. and Folkman,J. (1993) Betacellulin: a mitogen from pancreatic β cell tumors. *Science*, **259**, 1604–1607.

Sibilia,M. and Wagner,E.F. (1995) Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science*, **269**, 234–238.

Sliwkowski,M.X. *et al.* (1994) Coexpression of *erb*B2 and *erb*B3 proteins reconstitutes a high affinity receptor for heregulin. *J. Biol. Chem.*, **269**, 14661–14665.

Soltoff,S.P., Carraway,K.L., Prigent,S.A., Gullick,W.G. and Cantley,L.C. (1994) ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. *Mol. Cell. Biol.*, **14**, 3550–3558.

Taverna,D., Groner,B. and Hynes,N.E. (1991) Epidermal growth factor receptor, platelet-derived growth factor receptor, and c-erbB-2 receptor activation all promote growth but have distinctive effects upon mouse mammary epithelial cell differentiation. *Cell Growth Differentiat.*, **2**, 145–154.

Threadgill,D.W. *et al.* (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science*, **269**, 230–234.

Tzahar,E. *et al.* (1994) ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all neu differentiation/heregulin isoforma. *J. Biol. Chem.*, **269**, 25226–25233.

Ullrich,A. *et al.* (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature*, **309**, 418–425.

van der Geer,P., Hunter,T. and Lindberg,R.A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol.*, **10**, 251–337.

Wada,T., Qian,X. and Greene,M.I. (1990) Intermolecular association of the $p185^{neu}$ protein and EGF receptor modulates EGF receptor function. *Cell*, **61**, 1339–1347.

Wallasch,C., Weiss,F.U., Niederfellner,G., Jallal,B., Issing,W. and Ullich,A. (1995) Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *EMBO J.*, **14**, 4267–4275.

Yamamoto,T., Ikawa,S., Akiyama,T., Semba,K., Nomura,N., Miyajima,N., Saito,T. and Toyoshima,K. (1986) Similarity of the protein encoded by the human c-*erbB*-2 gene to the epidermal growth factor receptor. *Nature*, **319**, 230–234.

Yarden,Y. and Schlessinger,J. (1987) Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. *Biochemistry*, **26**, 1443–1451.

Zhang,K., Sun,J., Liu,N., Wen,D., Chang,D., Thomason,A. and Yoshinaga,S.K. (1996) Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. *J. Biol. Chem.*, **271**, 3884–3890.

Received on October 14, 1996; revised on December 5, 1996